



EDGEWOOD CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1471

PILOT-SCALE PRODUCTION AND TESTING OF A RECOMBINANT STAPHYLOCOCCAL ENTEROTOXIN (SEB) TRIPLE MUTANT

Alena Calm

RESEARCH AND TECHNOLOGY DIRECTORATE

E. Randal Hofmann

Gabrielle Boyd

EXCET, INC.

Springfield, VA 22150-2519

Chris Mangaya

Kelley Betts

LEIDOS, INC.

Reston, VA 21010-6848

September 2017

Approved for public release: distribution unlimited.

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 h per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) XX-09-2017		2. REPORT TYPE Final		3. DATES COVERED (From - To) Mar 2010 – Dec 2011	
4. TITLE AND SUBTITLE Pilot-Scale Production and Testing of a Recombinant Staphylococcal Enterotoxin (SEB) Triple Mutant				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Calm, Alena (ECBC); Hofmann, E. Randal; Boyd, Gabrielle (Excet); Mangaya, Chris; and Betts, Kelley (Leidos)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Director, ECBC, ATTN: RDCB-DRB-M, APG, MD 21010-5424 Excet, Inc., 6225 Brandon Avenue, Suite 360, Springfield, VA 22150-2519 Leidos, Inc., 11955 Freedom Drive, Reston, VA 20190-5651				8. PERFORMING ORGANIZATION REPORT NUMBER ECBC-TR-1471	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Joint Program Executive Office for Chemical and Biological Defense, Joint Project Manager Guardian, Defense Biological Products Assurance Office, 110 Thomas Johnson Drive, Suite 250, Frederick, MD 21702-4400				10. SPONSOR/MONITOR'S ACRONYM(S) JPEO-CBD, JPM-G, DBPAO	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release: distribution unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: The use of chemically or thermally inactivated biothreat toxins in detection methodologies can prove problematic when the resulting toxoids become attenuated or altered to the point that they are no longer recognizable by the antibody-based test methods for which they were intended. Recombinant technology was used to assess whether a nontoxic surrogate could be developed that retains the relative epitopes for antibody-based detection platforms. Here, three small batches of staphylococcal enterotoxin B triple mutant (L45R, Y89A, and Y94A) were prepared for the Defense Biological Products Assurance Office (DBPAO) to demonstrate the reproducibility of recombinant antigen production. Yields of starting material and final purified product were determined. Post-production testing for these batches was performed to identify purity, homogeneity, and activity as determined by the DBPAO using a Meso Scale Discovery SEB SinglePlex PR2 electrochemiluminescence assay (Meso Scale Diagnostics; Rockville, MD). Test results revealed that this product demonstrates high levels of reproducibility and consistency in yield, purity, homogeneity, and antigenicity.					
15. SUBJECT TERMS Recombinant antigen production Staphylococcal enterotoxin variant (SEBv) Triple mutant Electrochemiluminescence (ECL) Affinity purification					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 24	19a. NAME OF RESPONSIBLE PERSON Renu B. Rastogi
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code) (410) 436-7545

Blank

PREFACE

The work described in this report was started in March 2010 and completed in December 2011.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release.

Blank

CONTENTS

1.	INTRODUCTION	1
2.	METHODS	2
2.1	Expression of SEBv in <i>E. coli</i>	2
2.2	Affinity Purification of SEBv	2
2.3	Concentration Analysis of SEBv	2
2.4	Assessment of Purity.....	3
2.5	Determination of Polydispersity and Hydrodynamic Radius Using Dynamic Light Scattering (DLS)	3
2.6	Determination of Antigenicity	3
3.	RESULTS	4
3.1	Expression and Purification of SEBv in <i>E. coli</i>	4
3.2	Purity Analysis of SEBv	4
3.3	Homogeneity and Dispersity.....	5
3.4	Activity of SEBv.....	6
4.	DISCUSSION	7
	LITERATURE CITED	9
	ACRONYMS AND ABBREVIATIONS	11
	APPENDIX: RADIUS HISTOGRAMS	13

FIGURES

1.	Experion system-generated gel image of SEBv stored at (A) 4 °C and (B) –80 °C, at time zero	5
2.	SEBv 4 and –80 °C ECL data plotted using GraphPad software	7

TABLES

1.	SEBv Production Data	4
2.	SEBv Production Data from DLS Analysis	6

PILOT-SCALE PRODUCTION AND TESTING OF A RECOMBINANT STAPHYLOCOCCAL ENTEROTOXIN (SEB) TRIPLE MUTANT

1. INTRODUCTION

The use of chemically or thermally inactivated biothreat toxins in detection methodologies can prove problematic when the resulting toxoids become attenuated or altered to the point that they are no longer recognized by the antibody-based test methods for which they were intended. To address this problem, recombinant technology has been applied to assess whether a nontoxic surrogate can be developed that retains the relative epitopes for antibody-based detection platforms.

Staphylococcal enterotoxins are members of a family of more than 20 different, functionally related staphylococcal and streptococcal exotoxins that share sequence homology.¹⁻³ These bacterial proteins are known to be pyrogenic and are connected to significant human diseases such as food poisoning and toxic shock syndrome. For the most part, these toxins are produced by *Staphylococcus aureus*, although other species are also enterotoxigenic.¹⁻³ Staphylococcal enterotoxins are broadly classified as superantigens, which have the ability to stimulate large populations of T-cells (~20–30%), leading to the production of a cytokine bolus.¹⁻³ At least 20 serologically distinct staphylococcal superantigens have been described that include staphylococcal enterotoxin A through V (SEA, SEB, etc.) and toxic shock syndrome toxin-1 (TSST-1). SEA, SED, and SEE share 70–90% sequence homology, whereas they share only 40–60% sequence homology with SEB, SEC, and TSST-1.¹⁻³ Staphylococcal enterotoxins have mature lengths of ~220–240 amino acids, depending on the toxin, and their molecular sizes are ~25 kDa, on average. Staphylococcal enterotoxins have significant sequence variability, but when they are folded, they have similar three-dimensional structures.

SEB is soluble in water and is quite stable to heat, proteolytic digestion, and a wide pH range. These characteristics make SEB easy to produce and distribute.⁴ The effective dose of SEB for 50% of the population (ED₅₀) is 0.0004 µg/kg, and the lethal dose for 50% of the population (LD₅₀) is only 0.02 µg/kg;² therefore, inhalation of large quantities of SEB can lead to death by septic shock. Although SEB is responsible for only ~10% of food poisoning cases, in aerosol form, SEB is extremely toxic: the lethal dose for a 70 kg adult is only 1.4 µg.¹ Because of the low quantities needed to debilitate soldiers on the battlefield, SEB is considered a major biological threat. The relative ease of SEB transmission through food and water, as well as the toxin's ability to be aerosolized, make the need for detection assays extremely important. Several techniques are currently available for SEB detection.^{1,4,5}

SEB toxicity is mediated through its interaction with the major histocompatibility complex (MHC) class II on target cells, which results in widespread leukocyte proliferation and cytokine release. Single mutations of key residues in the polar pocket (Y89A and Y115A) or the hydrophobic binding loop (L45R) eliminate binding to the MHC class II molecule, human leukocyte antigen–antigen D related (HLA-DR1). Previous studies of a recombinant SEB triple mutant (L45R, Y89A, and Y94A) produced in *Escherichia coli* cells demonstrated a lack of super-antigen activity in rhesus monkey leukocyte cultures from animals immunized with the

attenuated recombinant vaccine.⁶ Furthermore, the SEB triple-mutant vaccine (SEBv) showed protection in both mice and Rhesus monkeys when challenged with wild-type SEB.⁶

To investigate whether recombinant SEB triple-mutant protein antigen could be reproducibly manufactured, three small batches of recombinant SEBv that contained the three mutations (L45R, Y89A, and Y94A) were prepared by the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD) using expression constructs provided by Ellen Goldman and George Anderson at the Naval Research Laboratory (NRL; Washington, DC).⁷ Following standard affinity purification protocols, we characterized the lots of recombinant protein for biophysical and immunological properties. Our test results demonstrated high reproducibility and consistency in yield, purity, homogeneity, and antigenicity.

2. METHODS

2.1 Expression of SEBv in *E. coli*

Eleven nanograms of pET15b plasmid DNA containing the coding region for SEBv (provided by NRL) was transformed into BL21 (DE3) pLysS electrocompetent *E. coli*. For each batch, 10 mL of Luria broth with 100 µg/mL of carbenicillin was inoculated from a single colony. The cultures grew overnight at 37 °C and were scaled up to six 500 mL cultures for a total of 3 L per lot in Novagen Overnight Express instant terrific broth media (EMD Millipore; Billerica, MA) containing 100 µg/mL of carbenicillin. The *E. coli* batches were cultured for 20 h. The cell mass was collected via centrifugation to create a cell paste and frozen at –80 °C until purification.

2.2 Affinity Purification of SEBv

To purify recombinant SEBv, the cell paste was thawed and resuspended in wash buffer (20 mM sodium phosphate, 20 mM imidazole, and 500 mM sodium chloride, pH 8.0) in a 1:5 weight–volume ratio. Cells were lysed using an M-110P microfluidizer (Microfluidics; Westwood, MA) at 20,000 psi. The lysate was clarified via centrifugation at 40,000 ×g for 2 h, and the supernatant was frozen at –80 °C. For the sake of reproducibility, the other lots were treated in the same manner. Supernatants were thawed and applied to a 1 mL nickel affinity column (GE Healthcare; Piscataway, NJ) at 0.4 mL/min on an ÄKTAexpress system (GE Healthcare). The column was washed with 20 column volumes of wash buffer to remove any loosely bound material, and SEBv was eluted with 500 mM imidazole. Peak fractions were detected by the ÄKTAexpress system, sent to an in-line desalting column (GE Healthcare), and eluted with phosphate-buffered saline (PBS; Fisher Scientific; Pittsburgh, PA).

2.3 Concentration Analysis of SEBv

The SEBv concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific; Waltham, MA). The absorbance at 280 nm was recorded, and the concentration was calculated using a 1.1 extinction coefficient (as calculated with the ProtParam tool on the ExPASy server [Expasy.org]) and the sequence for SEBv.

2.4 Assessment of Purity

Molecular weight and purity data were collected using an Experion automated electrophoresis system (Bio-Rad; Hercules, CA). For Experion analysis, each of the SEBv samples was processed using the validated procedure specified in the *Bio-Rad Experion Pro260 Analysis Kit* manual.⁸ Briefly, a Pro260 microfluidic chip was prepared by adding 12 μ L of Pro260 gel and gel stain to the designated wells. The chip was then placed on the priming station and primed for 1 min at the medium (B) pressure setting. The priming filled the fluidic channels with gel, which was used by the instrument to form a barrier between samples during the run. The sample was reduced with 2-mercaptoethanol (Bio-Rad), denatured in the kit-provided sample buffer at 95 °C, and then applied to the primed chip. The chip was placed in the instrument, and the lid was closed, lowering the sample needles into the wells. The instrument was operated using the Experion software, and each chip required 30 min to complete. All samples were run in triplicate, and analysis was performed using the Experion software.

2.5 Determination of Polydispersity and Hydrodynamic Radius Using Dynamic Light Scattering (DLS)

For DLS analysis, five 20 μ L aliquots of the SEBv samples were placed into a quartz 384 well plate (Wyatt Technology Corporation; Santa Barbara, CA) and centrifuged for 2 min at 1000 $\times g$ to remove trapped air bubbles. Mineral oil (Sigma-Aldrich; St. Louis, MO) was applied to the top of each sample to prevent sample evaporation, and the plate was then placed into a DynaPro temperature-controlled plate reader (Wyatt Technology). Each well was scanned 10 times for 5 s each at 25 °C. Wyatt Technology Dynamics software was used to obtain triplicate results, which were averaged to provide measurements of polydispersity, hydrodynamic radius, percent mass, and molecular weight for each sample.

2.6 Determination of Antigenicity

The reactivity to SEB-specific antibodies in an immunoassay was measured using the Meso Scale Discovery (MSD) PR2 model 1800 electrochemiluminescence (ECL) detection system (Meso Scale Diagnostics; Rockville, MD). Defense Biological Products Assurance Office (DBPAO) provided the MSD singleplex anti-SEB plates. SEBv samples were diluted in PBS containing 0.1% Triton X-100 solution (PBS-T). Serial dilutions were generated for assay linearity determinations, estimated limits of detection (LODs), signal-to-noise ratios (S/Ns), and assay variability. Assays were performed in accordance with the MSD protocol and using the detection antibody solution, diluent, and read buffer that were provided with the assay. Background signal was determined from the average of four blank (buffer only) samples, and test sample values greater than 3 standard deviations above background were considered significant.

3. RESULTS

3.1 Expression and Purification of SEBv in *E. coli*

SEBv with a C-terminal histidine tag was grown as three separate batches on separate days and purified using nickel affinity purification. The wet mass of the bacterial paste averaged 34 ± 3 g of starting material. The final yield for each 3 L batch was 11.2 ± 0.3 mg, for an average of 3.8 ± 0.1 mg/L of culture (Table 1). The protein concentration as estimated by the ÄKTAexpress software was confirmed using a NanoDrop spectrophotometer. These concentrations were 1.05, 1.25, and 1.23 mg/mL for lots 10052015-01, 10062015-01, and 10072015-01, respectively.

Table 1. SEBv Production Data

Lot Number	Pellet Weight (g)	Volume* (mL)		SEBv* Concentration (mg/mL)	Yield	
		Load	Elution*		Total SEBv* (mg)	SEBv/L Culture (mg/L)
10052015-01	33.6	210	10.0	1.1	11.0	3.7
10062015-01	31.3	185	10.1	1.1	11.1	3.7
10072015-01	37.2	210	10.5	1.1	11.6	3.9

*Data generated from ÄKTAexpress software.

3.2 Purity Analysis of SEBv

Each lot of SEBv was analyzed for purity using the Experion lab-on-a-chip Pro260 system. SEBv is a 31 kDa protein and should be visible as a single band on the electronic gel image. Figure 1A shows that all three lots were greater than 95% pure, and the molecular weight was between 32 and 33 kDa when stored at -4 °C. Figure 1B demonstrates that all three production lots were greater than 95% pure, and the molecular weight was between 32 and 33 kDa when stored at -80 °C. The cumulative results demonstrate that the production, extraction, and purification methods yielded a SEBv protein that was consistently pure across different production runs.

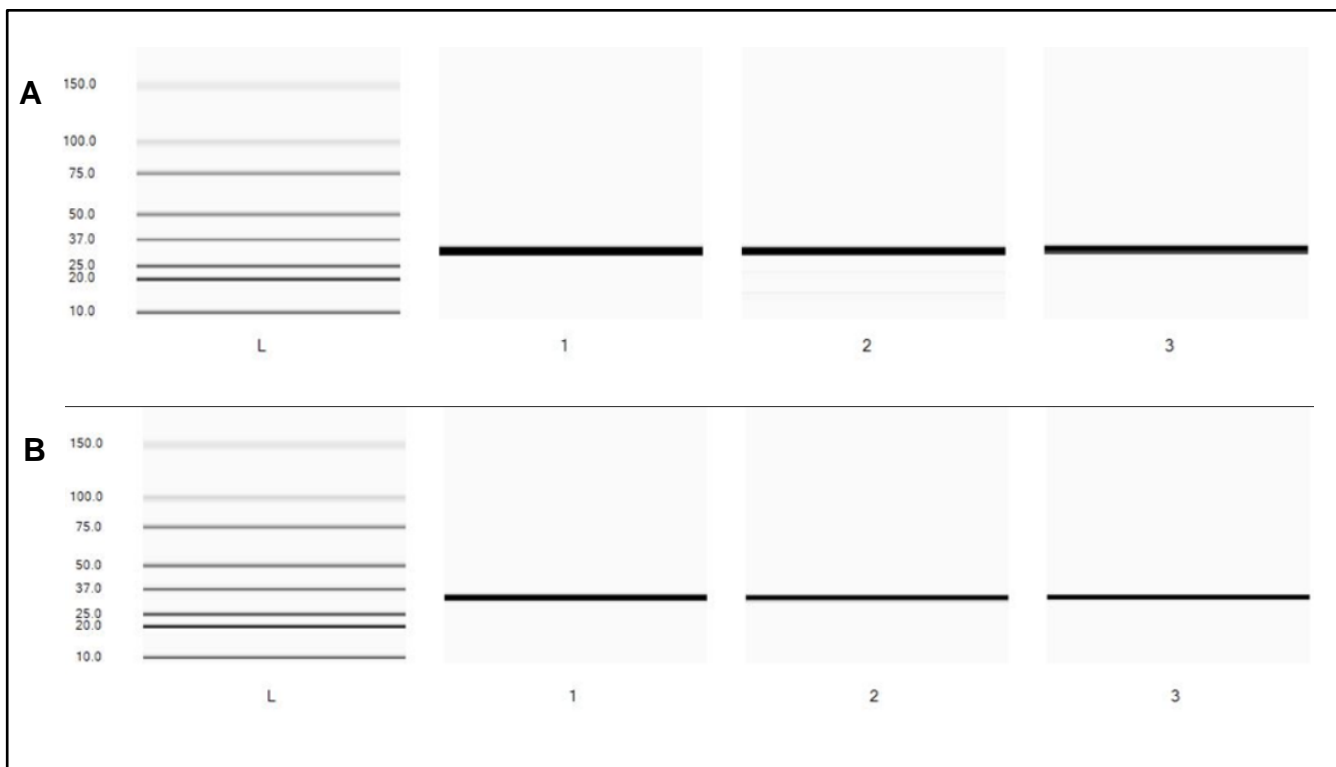


Figure 1. Experion system-generated gel image of SEBv stored at (A) 4 °C and (B) –80 °C, at time zero. Lanes 1–3 represent lots 10052015-1, 10062015-1, and 10072015-1, respectively. The ladder (far left) is the molecular weight standard.

3.3 Homogeneity and Dispersity

DLS was used to interrogate each lot of SEBv to determine the physical properties of SEBv in solution. DLS identifies protein–protein interactions and the state of aggregation in solution. All data analyses were performed using DynaPro software (Wyatt Technology). Based on the globular protein prediction algorithm in the DynaPro software, SEBv was estimated to have a hydrodynamic radius of 2.6 nm. The correlation graphs for all samples exhibited a quick decay and a smooth sigmoidal curve, which are indicative of small, uniform particle size (data not shown). Table 2 summarizes the data gathered from the DLS analyses. In general, there were no significant differences in the physical properties of the different production lots stored at different temperatures. The average radius for all three lots was 2.8 nm, and the standard deviations were 1.4 and 1.6 nm for 4 and –80 °C, respectively. The average peak percentages of polydispersity were 17.6 ± 7.5 and $15.2 \pm 4.5\%$ at 4 and –80 °C, respectively. This data represented >99.9% of the mass measured and fell within the expected range based on the molecular weight of SEBv. The corresponding graphs are provided in the appendix.

Table 2. SEBv Production Data from DLS Analysis

Lot Number	Temperature (°C)	Radius (nm)	Peak Polydispersity (%)	Peak Mol Wt (kDa)	Peak Mass (%)
10052015	4	2.8002	12.385	37.546	99.971
10062015	4	2.7377	14.114	36.102	99.985
10072015	4	2.7438	13.401	35.742	99.966
10052015	−80	2.6253	11.87	32.262	99.947
10062015	−80	2.7438	13.401	35.742	99.966
10072015	−80	2.9431	20.244	42.159	99.973

3.4 Activity of SEBv

Each lot of SEBv was tested for activity using the MSD ECL assay for staphylococcus enterotoxin B. The plates were read on the PR2 instrument, and results were analyzed using GraphPad Prism software (GraphPad Software; San Diego, CA). ECL assay indicated that the 4 and −80 °C samples exhibited the same activity, and there was no significant difference in activity between the three lots (Figure 2).

All three lots of SEBv stored at −80 °C were detected at concentrations as low as 0.5 pg/mL (LOD) with an average S/N of 1.8. Two of the lots stored at 4 °C had an LOD of 0.5 pg/mL, and lot 10072015-01 was detected as low as 0.1 pg/mL. The average S/N for these three lots was 1.6. These LODs were in line with the confirmed LOD of a pilot batch that was used to characterize the MSD singleplex plates on the PR2 model 1800 ECL system for DBPAO.

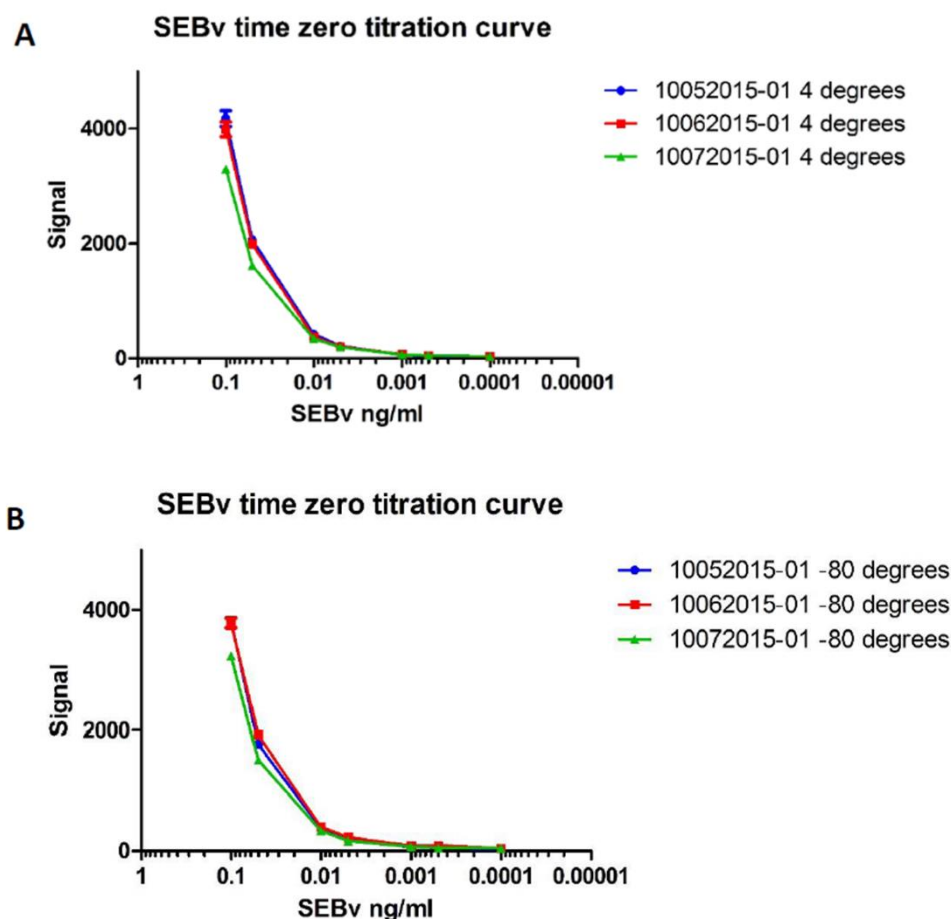


Figure 2. SEBv 4 and -80°C ECL data plotted using GraphPad software. (A) SEBv 4°C ECL data. The x axis shows a titration of the SEBv antigen, and the signal is shown on the y axis. (B) SEBv -80°C ECL data. The x axis shows a titration of the SEBv antigen, and the signal is shown on the y axis. Error bars represent four replicates at each concentration.

4. DISCUSSION

To demonstrate the reproducibility of recombinant antigen production technology, three small batches of SEBv were prepared for the DBPAO. Post-production testing included DLS, Experion analysis, concentration analysis, and an activity assay. We found that when we followed a particular standard operating procedure for culturing the host bacterial strain, an affinity purification yielded a consistent and highly reproducible product. Our recombinant protein samples were consistently produced at high concentrations ($>1\text{ mg/mL}$) at purities exceeding 95%. The product demonstrated good solubility and homogeneity with no aggregation. Furthermore, the antigenicity was comparable to active wild-type toxin (data not shown). These lots are currently undergoing shelf-life studies that utilize the same conformance testing at storage conditions of 4 and -80°C . A separate report will be provided for those studies.

Blank

LITERATURE CITED

1. Ulrich, R.G.; Sidell, S.; Taylor, T.J.; Wilhelmsen, C.L.; Franz, D.R. Staphylococcal Enterotoxin B and Related Pyrogenic Toxins. In *Textbook of Military Medicine: Medical Aspects of Chemical and Biological Warfare*; Zajtchuk, R., Bellamy, R.F., Eds.; Part I; Office of the Surgeon General, U.S. Department of the Army: Washington, DC, 1997; pp 621–630.
2. Balaban, N.; Rasooly, A. Staphylococcal Enterotoxins. *Int. J. Food Microbiol.* **2000**, *61*, 1–10.
3. Krakauer, T.; Stiles, B.G. The Staphylococcal Enterotoxin (SE) Family: SEB and Siblings. *Virulence* **2013**, *4*, (8), 759–773.
4. Kijek, T.M.; Rossi, C.A.; Moss, D.; Parker, R.W.; Henschel, E.A. Rapid and Sensitive Immunomagnetic-Electrochemiluminescent Detection of Staphylococcal Enterotoxin B. *J. Immunol. Methods* **2000**, *236*, (1–2), 9–17.
5. McLauchlin, J.; Narayanan, G.L.; Mithani, V.; O'Neill, G. The Detection of Enterotoxins and Toxic Shock Syndrome Toxin Genes in *Staphylococcus aureus* by Polymerase Chain Reaction. *J. Food Prot.* **2000**, *63*, (4), 479–488.
6. Ulrich, R.G.; Olson, M.A.; Bavari, S. Development of Engineered Vaccines Effective against Structurally Related Bacterial Superantigens. *Vaccine* **1998**, *16* (19), 1857–1864.
7. Anderson, G.P.; Legler, P.M.; Zabetakis, D.; Goldman, E.R. Comparison of Immunoreactivity of Staphylococcal Enterotoxin B Mutants for Use as Toxin Surrogates. *Anal. Chem.* **2012**, *84*, (12), 5198–5203.
8. *Instruction Manual, Experion Pro260 Analysis Kit*, Rev C; catalog no. 10000975; Bio-Rad Laboratories: Hercules, CA, 2010.

Blank

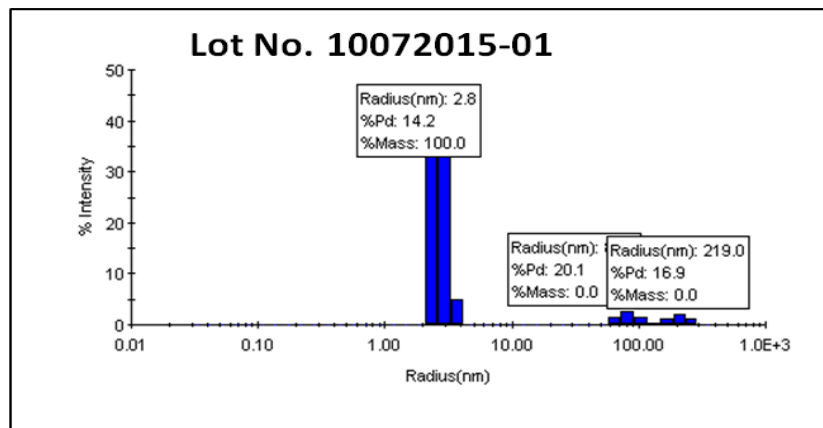
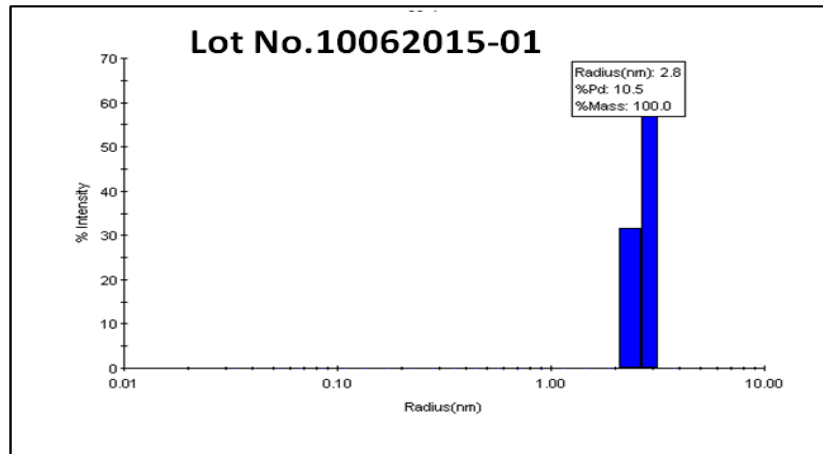
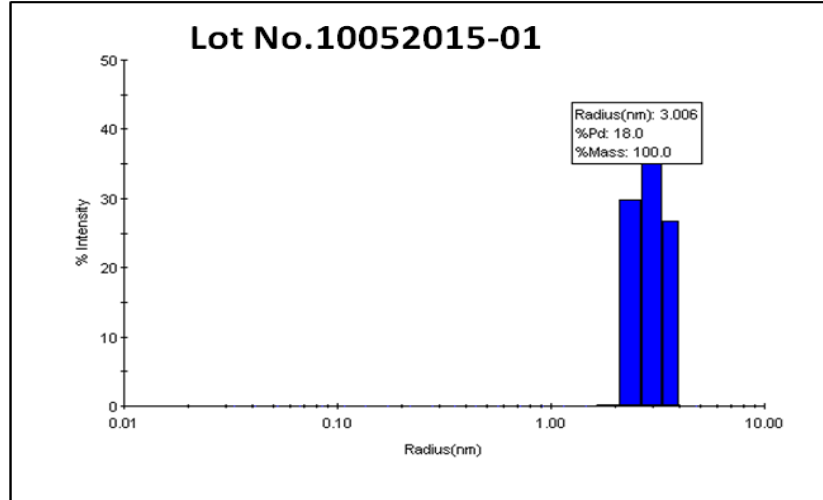
ACRONYMS AND ABBREVIATIONS

DBPAO	Defense Biological Products Assurance Office
DLS	dynamic light scattering
ECL	electrochemiluminescence
ED ₅₀	effective dose for 50% of the population
LD ₅₀	lethal dose for 50% of the population
LOD	limit of detection
MHC	major histocompatibility complex
MSD	Meso Scale Discovery
NRL	Naval Research Laboratory
PBS	phosphate-buffered saline
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B
SEBv	staphylococcal enterotoxin triple-mutant variant
SEC	staphylococcal enterotoxin C
SED	staphylococcal enterotoxin D
SEE	staphylococcal enterotoxin E
S/N	signal-to-noise ratio
TSST-1	toxic shock syndrome toxin-1

Blank

APPENDIX

RADIUS HISTOGRAMS



DISTRIBUTION LIST

The following individuals and organizations were provided with one Adobe portable document format (pdf) electronic version of this report:

U.S. Army Edgewood Chemical
Biological Center (ECBC)
RDCB-DRB-M
ATTN: Calm, A.
Hofmann, E.
Thompson, R.

Defense Threat Reduction Agency
J9-CBS
ATTN: Graziano, A.

Department of Homeland Security
RDCB-PI-CSAC
ATTN: Negron, A.
DHS-S&T-RDP-CSAC
ATTN: Strang, P.

G-3 History Office
U.S. Army RDECOM
ATTN: Smart, J.

Office of the Chief Counsel
AMSRD-CC
ATTN: Upchurch, V.

ECBC Rock Island
RDCB-DES
ATTN: Lee, K.
RDCB-DEM
ATTN: Grodecki, J.

ECBC Technical Library
RDCB-DRB-BL
ATTN: Foppiano, S.
Stein, J.

Defense Technical Information Center
ATTN: DTIC OA

